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ORIGINAL ARTICLE

Cytotoxic effects of *Agrimonia eupatoria* L. against cancer cell lines *in vitro*

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Abstract The study was conducted to evaluate anti-tumor (human cervical cancer; HeLa and Rhabdomyosarcoma; RD cell lines and a primary cell culture; mouse embryo fibroblast; MEF) potentials of *Agrimonia eupatoria* L. extracts (aqueous and methanol). Five concentrations (6.0, 12.0, 24.0, 48.0 and 96.0 μg/ml) of each plant extract were assessed through either three incubation time periods (24, 48 and 72 h) for HeLa and RD cell lines, or one incubation time period (48 h) for MEF cells. The results revealed that the five concentrations of plant extracts showed anti-tumor properties in a concentration-dependent manner, and the methanol extract recorded better values of percentage of growth inhibition (PGI) than aqueous extract in HeLa and RD cell lines, while, less PGI values were recorded in the MEF cells. Among these concentrations, 96.0 μg/ml was the most effective in producing PGI in RD and HeLa cancer cell lines for the three investigated time periods.

1. Introduction

Herbal medicines derived from plants are being increasingly utilized to treat a wide variety of diseases in folkloric medicine and accordingly, interest in pharmacological evaluation of various plants shows a progress worldwide, although a little knowledge is available about their mechanism of action (Gupta et al., 2004). *Agrimonia eupatoria* (Rose Family: *Rosaceae*) is a further interest of medicinal plants. The plant is known as agrimony in the English literature, and it is used in folkloric medicine to treat a wide range of ailments; for instance, eye infections, diarrhea and disorders of gall bladder, liver and kidneys (Duke, 2002). However, experimental studies have demonstrated several other biological and pharmaceutical potentials; for instance, anti-mutagenic and anti-tumor (Horikawa et al., 1994), hepatoprotective (Park et al., 2004), anti-viral (Kwon et al., 2005), anti-bacterial (Bae and Sohn, 2005) and anti-oxidant and anti-inflammatory (Correia et al., 2007) effects. This is reasoned by the fact that *A. eupatoria* is rich in chemical constituents (flavonoids, tannins, aromatic acids, triterpenes, coumarins, terpenoids, glycosides, and vitamins B and K) that can mediate such potentials (Xu et al., 2005).

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With respect to anti-mutagenic and anti-tumor potentials, Koshiura et al. (1985) was the first who investigated the anti-tumor activity of another species (*Agrimonia pilosa*). In this study, effects of a methanol extract from roots of the plant on several transplantable rodent tumors were evaluated. Their results indicated that the roots of the plant contain some anti-tumor constituents, and they also suggested that the possible mechanisms of the anti-tumor activity may be related to some host-mediated actions and/or a direct cytotoxicity. Based on such finding, Miyamoto et al. (1988) investigated the effect of agrimoniin, a tannin contained in *A. pilosa*, on ascites type and solid type rodent tumors. Their results indicated that agrimoniin is a potent anti-tumor tannin and they suggested that the anti-tumor effect may be due to this tannin, which can enhance the immune response of the host animals through the actions on tumor cells and some cells of the immune system. In 1992, it was found that agrimoniin is able to induce interleukin (IL)-1 production *in vitro* (human peripheral blood mononuclear cells) and *in vivo* (mouse adherent peritoneal exudate cells), and accordingly the anti-tumor effects can be justified (Murayama et al., 1992). Anti-mutagenic and anti-carcinogenic effects of *A. pilosa* were further confirmed *in vitro* against the environmental mutagens and carcinogens benzo[a]pyrene, 1,6-dinitropyrene and 3,9-dinitrofluoranthene (Horikawa et al., 1994).

As presented, studies have been engaged in assessing the potentials of *A. pilosa*, while *A. eupatoria* has not been investigated extensively; therefore the present investigation came to evaluate cytotoxic effects of *A. eupatoria* aqueous and methanol extracts *in vitro* against two cancer cell lines (Human rhabdomyosarcoma; RD and human cervical cancer; HeLa) and a primary cell culture (mice embryo fibroblast; MEF).

2. Materials and methods

2.1. Plant collection and identification

The plant taxonomist Professor Dr. Ali Al-Mosawy (Department of Biology, College of Science, University of Baghdad) identified the plant *A. eupatoria*, which was collected in July 2008 from mountain regions (Binqulat village) surrounding the city of Erbil (around 400 km North the capital Baghdad).

2.2. Extraction

Dried aerial parts of *A. eupatoria* (leaves, stem and flowers) were powdered using a coffee grinder for 5 min, and then extracted with two types of solvents (distilled water or methanol). In both cases, 50 grams of the processed plant was extracted in 250 ml of the solvent using the Soxhlet apparatus and the source of heating was a warm water bath (45 °C). The obtained extract solution was then evaporated at 45 °C using a rotary evaporator, and the resultant crude extract was frozen at −20 °C until use to prepare the required concentrations (Arokiyaraj et al., 2007).

2.3. Cancer cell lines and normal cells

RD cell line was on passage number 195, while HeLa cell line was on passage number 185. Both cell lines were supplied by Al-Nahrain Research Centre (Al-Nahrain University), while MEF cells were prepared from a pregnant mouse at a gestation period of 11–13 days, following the instructions of Freshney (2000).

2.4. Growth inhibition assessment

Laboratory assessment of growth inhibition was carried out according to a method presented by Freshney (2001). The cells were supplemented as monolayer attached cells in Falcon culture flasks (25 ml) containing RPMI-1640 medium (Sigma, USA). The cells were washed with PBS, and then 1 ml of trypsin–versene solution (Sigma, USA; BDH, England) was added with a gentle shaking until cells were detached from the flask surface. Such manipulation was carried out with the aid of a phase contrast inverted microscope. Then, the contents of the flask were transferred to another flask and incubated at 37 °C (supplemented with 5% CO_2) for 15 min (sub-culture), and then cell number was adjusted to 1×10^6 cell/ml. At the same time, viability was assessed using a dye-exclusion test (trypan blue stain; Pharmacia fine chemical, Sweden), and it was always greater than 96%. Such cells were seeded in wells of a 96-well tissue culture plate, and the plate was incubated overnight at 37 °C (supplemented with 5% CO_2). The day after, the wells were examined to inspect the formation of a cell monolayer, and then five concentrations (6.0, 12.0, 24.0, 48.0 and 96.0 μg/ml) of each plant extract were prepared and the solvent was RPMI-1640 medium. In the case of methanol extract, it was first dissolved in drops of DMSO, and then the volume was made-up with RPMI-1640 medium. Both extract solutions were filter-sterilized (0.22 μm Millipore filter). In each well, 50 μl of each concentration was pipetted and the plate was wrapped with a cling film and incubated at 37 °C (supplemented with 5% CO_2). In this regard three plates were prepared to cover three incubation time periods; 24, 48 and 72 h, except MEF cells, in which the incubation period was 48 h. After the end of each incubation time period, the medium in each well was discarded and wells were washed once with PBS, and then 0.8 ml of neutral red solution (Sigma, USA) was added to each well. The plate was re-incubated at 37 °C for 2 h. After incubation, neutral red solution was discarded and each well was washed once with PBS, and in each well, 0.1 ml of phosphate buffered-ethanol (0.1 M NaH_2PO_4-ethanol; 1:1) was added. The wells were read using ELISA reader at a wave length of 492 nm, and absorbance was recorded. Percentage of growth inhibition (PGI) of the tested materials was calculated using the following equation:

$$\text{Growth inhibition}(\%) = \left(\frac{\text{Control Absorbance} - \text{Treated Absorbance}}{\text{Control Absorbance}}\right) \times 100$$

2.5. Statistical analysis

For each evaluation, the mean of three observations was obtained and differences between means were assessed by least significant difference (LSD) and Duncan test, using the statistical package SPSS version 13.0. The difference was considered significant when probability (P) $\leqslant 0.05$.

Table 1 Growth inhibition effects of *Agrimonia eupatoria* aqueous extract on RD and HeLa cell lines after 24 h incubation.

Extract concentration (μg/ml)	Percentage of growth inhibition (Mean ± SE)*		LSD probability
	RD cell line	HeLa cell line	
6	15.07 ± 0.43[a]	20.97 ± 0.24[a]	Not significant
12	21.37 ± 1.72[b]	26.70 ± 0.40[b]	Not significant
24	23.80 ± 4.33[b]	27.25 ± 2.63[b]	Not significant
48	23.97 ± 4.33[b]	33.03 ± 0.88[c]	0.007
96	35.77 ± 6.04[c]	33.37 ± 1.92[c]	Not significant

* Different letters: significant difference ($P \leqslant 0.5$) between means of the columns.

Table 2 Growth inhibition effects of *Agrimonia eupatoria* aqueous extract on RD and HeLa cell lines after 48 h incubation.

Extract concentration (μg/ml)	Percentage of growth inhibition (Mean ± SE)*		LSD probability
	RD cell line	HeLa cell line	
6	42.00 ± 4.61[a]	36.33 ± 2.67[a]	Not significant
12	42.00 ± 4.61[a]	53.33 ± 2.67[a]	Not significant
24	53.00 ± 3.00[b]	53.33 ± 2.67[a]	Not significant
48	53.00 ± 3.00[b]	59.67 ± 3.00[b]	Not significant
96	67.00 ± 9.81[c]	72.67 ± 16.33[c]	Not significant

* Different letters: significant difference ($P \leqslant 0.5$) between means of the columns.

Table 3 Growth inhibition effects of *Agrimonia eupatoria* aqueous extract on RD and HeLa cell lines after 72 h incubation.

Extract concentration (μg/ml)	Percentage of growth inhibition (Mean ± SE)*		LSD probability
	RD cell line	HeLa cell line	
6	57.33 ± 1.20[a]	60.00 ± 0.58[a]	Not significant
12	58.67 ± 0.33[a]	65.67 ± 0.67[b]	Not significant
24	60.33 ± 1.45[a]	69.00 ± 0.58[c]	0.026
48	70.00 ± 1.53[b]	75.33 ± 0.67[d]	Not significant
96	73.00 ± 0.58[b]	84.00 ± 0.58[e]	Not significant

* Different letters: significant difference ($P \leqslant 0.5$) between means of the columns.

3. Results

3.1. Aqueous extract (24 h of incubation)

There was a gradual increase in the value of PGI as the concentration of the extract was increased (15.07, 21.37, 23.80, 23.97 and 35.77% for the concentrations 6.0, 12.0, 24.0, 48.0 and 96.0 μg/ml, respectively) against RD cells. The 1st and 5th concentrations showed a significant difference ($P \leqslant 0.05$) in comparison with the 2nd, 3rd and 4th concentrations, and the latter concentrations showed no significant differences between them. A similar observation was made in HeLa cells, but the 4th concentration showed a significant difference ($P \leqslant 0.05$) in comparison with the 1st, 2nd and 3rd concentrations. This concentration also showed a significant difference ($P = 0.007$) in the PGI value when a comparison was made between HeLa (33.03%) and RD (23.97%) cells (Table 1).

3.2. Aqueous extract (48 h of incubation)

The five concentrations of aqueous extract were effective in reducing the growth of both cell lines, and almost the effect was similar (no significant differences between them) on RD and HeLa cells. The effect was concentration-dependent, and accordingly the 5th concentration recorded the highest PGI, which was 67% in RD cells and 72% in HeLa cells. These two values were significantly different ($P \leqslant 0.05$) in comparison with PGI values of the other concentrations of the same cell line (Table 2).

3.3. Aqueous extract (72 h of incubation)

Much more growth reducing effects were observed after 72 h of incubation, and the 5th concentration was the most efficient on both cell lines, in which no significant differences were recorded between them in all concentrations, with the exception of 24 μg/ml, which showed a significantly ($P = 0.026$) higher PGI in HeLa cells (69%) than the PGI in RD cells (60.33%) (Table 3).

3.4. Methanol extract (24 h of incubation)

The first four concentrations recorded a PGI range of 13–25.33% in RD cell line, while such range in HeLa cell line was 14.00–30.67%. The range exceeded dramatically at the 5th concentration (96 μg/ml), but the PGI in RD cell line was significantly ($P = 0.001$) higher than that of HeLa cell line (80.00% *vs.* 53.67%). A further significant difference ($P = 0.013$) was observed at the concentration of 12 μg/ml, in which the PGI in RD cell line was higher than that of HeLa cell line (25% *vs.* 16%) (Table 4).

Table 4 Growth inhibition effects of *Agrimonia eupatoria* methanol extract on RD and HeLa cell lines after 24 h incubation.

Extract concentration(μg/ml)	Percentage of growth inhibition (Mean ± SE)*		LSD probability
	RD cell line	HeLa cell line	
6	13.00 ± 1.00[a]	14.00 ± 1.15[a]	Not significant
12	25.00 ± 0.58[b]	16.67 ± 0.67[a]	0.013
24	24.00 ± 0.57[b]	27.67 ± 1.45[b]	Not significant
48	25.33 ± 0.33[b]	30.67 ± 3.67[b]	Not significant
96	80.00 ± 0.58[c]	53.67 ± 0.67[c]	0.001

* Different letters: significant difference ($P \leqslant 0.5$) between means of the columns.

Table 5 Growth inhibition effects of *Agrimonia eupatoria* methanol extract on RD and HeLa cell lines after 48 h incubation.

Extract concentration(μg/ml)	Percentage of growth inhibition (Mean ± SE)*		LSD probability
	RD cell line	HeLa cell line	
6	24.00 ± 2.00[a]	27.33 ± 1.45[a]	Not significant
12	27.33 ± 4.70[a]	33.67 ± 3.38[a]	Not significant
24	32.67 ± 0.66[a]	34.67 ± 6.56[a]	Not significant
48	36.67 ± 1.67[b]	36.67 ± 5.90[b]	Not significant
96	82.00 ± 1.00[c]	84.00 ± 1.00[c]	Not significant

* Different letters: significant difference ($P \leqslant 0.5$) between means of the columns.

Table 6 Growth inhibition effects of *Agrimonia eupatoria* methanol extract on RD and HeLa cell lines after 72 h incubation.

Extract concentration(μg/ml)	Percentage of growth inhibition (Mean ± SE)*		LSD probability
	RD cell line	HeLa cell line	
6	13.50 ± 0.29[a]	58.00 ± 7.94[a]	0.001
12	27.00 ± 2.08[b]	62.00 ± 6.56[b]	0.001
24	30.33 ± 0.67[b]	63.00 ± 2.00[b]	0.001
48	33.00 ± 1.00[b]	65.00 ± 1.00[b]	0.001
96	90.00 ± 3.05[c]	87.00 ± 2.65[c]	Not significant

* Different letters: significant difference ($P \leqslant 0.5$) between means of the columns.

Table 7 Growth inhibition effects of *Agrimonia eupatoria* extracts (aqueous and methanol) on MEF cell line after 48 h incubation.

Extract concentration (μg/ml)	Percentage of growth inhibition (Mean ± SE)*		LSD probability
	Aqueous extract	Methanol extract	
6	14.80 ± 0.72[a]	16.50 ± 1.44[a]	Not significant
12	10.90 ± 1.30[b]	15.63 ± 0.73[a]	0.01
24	12.40 ± 1.56[ab]	12.70 ± 1.24[b]	Not significant
48	13.00 ± 1.53[a]	14.13 ± 2.92[ab]	Not significant
96	14.00 ± 1.15[a]	10.53 ± 0.74[b]	0.01

* Different letters: significant difference ($P \leqslant 0.5$) between means of the columns.

3.5. Methanol extract (48 h of incubation)

Approximated values of PGI were recorded in the five tested concentrations for both cell lines, especially the 5th concentration in which the PGI was 82% and 84% for RD and HeLa cell lines, respectively (Table 5).

3.6. Methanol extract (72 h of incubation)

Again the 5th concentration recorded the highest PGI in RD and HeLa cell lines (90% and 87%, respectively) with no significant difference between them. However, the first four concentrations contradicted such scope, in which the PGI recorded significantly ($P = 0.001$) higher values in HeLa cells than in RD cells (Table 6).

3.7. Mouse embryonic fibroblast (MEF) Cell

The aqueous and methanol extracts recorded an approximated range of PGI against MEF cells (aqueous extract: 10.90–14.80%; methanol extract: 10.53–16.58%) with the exception of the concentration 12 μg/ml, in which the methanol extract

demonstrated a significantly ($P \leqslant 0.05$) increased PGI as compared with the PGI of the corresponding aqueous extract concentration (15.63% *vs.* 10.90%) (Table 7).

4. Discussion

The two extracts of *A. eupatoria* showed *in vitro* growth inhibition effects on the two cancer cell lines (RD and HeLa), while there was no effect on the growth on normal cells (MEF). Such selective effects were concentration-, as well as, incubation time period-dependent. With respect to concentration, the five assessed concentrations (6.0, 12.0, 24.0, 48.0 and 96.0 μg/ml) of each extract were evaluated after running preliminary assessments to determine the initial effective concentration. The start was with 1.0, 2.0 and 3.0 μg/ml, but they showed no effective growth inhibition against RD and HeLa cell lines or MEF cells. Therefore, five serial duplications of the third concentration were investigated. Among these concentrations, 96.0 μg/ml was the most effective in producing PGI in RD and HeLa cancer cell lines for the three investigated time periods. Such anti-proliferation effects can be justified in the ground of the extract chemical constituents.

Chemical analysis (data not shown) of *A. eupatoria* extracts (aqueous and methanol) revealed that the plant was positive for several secondary metabolites. Both aqueous and methanol extracts were positive for flavonoids, alkaloids, tannins and glycosides and negative for saponins. Flavonoids were further identified by thin layer chromatography (TLC), and as suggested by RF values of the separated extracts, the aqueous extract contained myricetin, azoleatin, vitexin and isoorientin, while the methanol extract contained kaempferol, quercetin, isorhamnetin and myricetin.

Flavonoid and tannin contents of the two extracts may be served as good anti-cancer agents (Huang et al., 2010). The mechanism of such anti-cancer cytotoxicity is not well understood, but the anti-oxidant potential of the two chemical constituents cannot be ignored (Russo et al., 2005), although the correlation between their anti-oxidative and anti-carcinogenic activities remains unclear (Tomisato et al., 2007). Among polyphenols is the tannin compound epigallocatechin-3-gallate, which has been demonstrated to possess anti-oxidative activity and to induce apoptosis in tumor cells (Chen et al., 2009). The apoptosis was found to occur through two main pathways; in the first death receptors are triggered, while the second pathway involves an intrinsic mitochondrial damage, which results in the release of cytochrome C and activation of caspase-9 (Gosse et al., 2005). Furthermore, polyphenolics can cause a down-regulation of factors that are necessary for cell-cycle progression, and this series of events lead to stoppage of cell cycle progression at the G1-S phase transition and thereby causing G0/G1 arrest and subsequent apoptotic cell death (Cilla et al., 2009). Accordingly, natural polyphenols have gained a great interest in pharmaceutical industry due to their demonstrated inhibitory activity against tumorigenesis, and a comparative study of Hakimuddin et al. (2008) proposed that total polyphenolics isolated from wine were more effective in reducing tumor growth as compared with a hydrophobic polyphenolic fraction isolated from the wine. Analyses of gene expression showed that genes belonging to signaling pathways were down-regulated in the induced tumor of grape polyphenol-treated mice.

Flavonoids are a further constituent of *A. eupatoria* extracts that might have exerted anti-tumor effects, and a study carried out by Aherne and O'Brien (2000) showed that flavonoids from different fruits and vegetables were capable to modulate H_2O_2-induced DNA damage in two cell line models (colon carcinoma and hepatic cell carcinoma). In agreement with such finding, it has been demonstrated that flavonoids are acting as a scavenging system, in addition to their role in arresting the DNA replication in S phase, and inducing apoptosis due to the inactivation of *BCL-2* gene as reported in different types of leukemias (Pellecchia and Reed, 2004). Furthermore, it has been suggested that polyphenolic fractions may affect the topological state of the DNA by increasing or decreasing the level of cleavage of DNA topoisomerases, especially DNA topoisomerase II; causing DNA topoisomerase II poisoning (Gonzalez de Mejia et al., 2006). The DNA topoisomerase II is a ubiquitous enzyme that regulates DNA unwinding and removes knots and tangles from the genetic material by creating transient breaks in the sugar phosphate back bone of the double helix (Fortune and Osheroff, 2000). Topoisomerases also maintain genomic integrity during this process by forming covalent attachments between active site residues and terminal DNA phosphates that are generated during the cleavage reaction (Champoux, 2001).

In the present study, it was also demonstrated that both extracts exerted a selective effect against investigated cell lines and normal cells, and in agreement with this finding, several *in vitro* studies have demonstrated the selective tumoricidal action of natural products, and the action occurred without harming the normal cell (Griffin et al., 2007); a finding which was also observed in the present study against the MEF cells. In this context, Weber (2009) demonstrated that the secondary metabolite flavonoids of *Scutellaria* were not only cytostatic but also cytotoxic to various human tumor cell lines *in vitro* and inhibit tumor growth *in vivo*, but most importantly, almost no or minor toxicity of these flavonoids against normal epithelial and normal peripheral blood and myeloid cells was observed.

In conclusion, the present plant extracts can be considered as an important source of natural products that have anti-cancer potentials, but it is too early to reach a final conclusion and further investigations are required to include further cell lines. Equally important, *in vivo* evaluation may certainly augment such potentials.

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